SUPPLEMENTAL MATERIALS

Altered Responsiveness to TGF-β and BMP and Increased CD45+ Cell Presence in Mitral Valves are Unique Features of Ischemic Mitral Regurgitation

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Supplemental Methods

Table I. MAJOR RESOURCES TABLE

Animals (in vivo and in vitro studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
Sheep	Archer Farms, Inc. Darlington MD	Dorset hybrid sheep	Male/Female	http://www.archerfarmsinc.com/sheep.asp

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)	Persistent ID / URL
P-SMAD2,3	Abcam	Ab188334	1.2µg/ml	GR3186711-5	https://www.abcam.com/smad2-phospho-s255-antibody-epr2856n-ab188334.html
SMAD2,3	Santa Cruz	Sc-133098	0.2µg/ml	#A0318	https://datasheets.scbt.com/sc-133098.pdf
P-SMAD1,5	Abcam	Ab92698	1µg/ml	GR275690-26	https://www.abcam.com/smad2-phospho-s255-antibody-epr2856n-ab188334.html
SMAD1,5	Abcam	Ab66737	0.6µg/ml	GR3186261-5	https://www.abcam.com/smad159-antibody-ab66737.html
α-tubulin	Sigma	T9026	4µg/ml	#029M4895V	https://www.sigmaaldrich.com/catalog/product/sigma/t9026
α-SMA	Abcam	Ab7817	0.3µg/ml	GR3356520-1	https://www.abcam.com/alpha-smooth-muscle-actin-antibody-1a4-ab7817.html
OPN	Abcam	Ab8448	1µg/ml	GR3286062-12	https://www.abcam.com/osteopontin-antibody-ab8448.html
Ki67	Abcam	Ab15580	1µg/ml	GR3362446-1	https://www.abcam.com/ki67-antibody-ab15580.html
CD45	Abcam	Ab10559	1µg/ml	GR3273058-3	https://www.abcam.com/cd45-antibody-hematopoietic-stem-cell-marker-ab10559.html

Data Availability

Description	Source / Repository	Persistent ID / URL
RNA sequencing data	NCBI's Gene Expression Omnibus	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE139921

Supplemental Methods

Ovine Model of IMR

All animal protocols were approved by the University of Pennsylvania's Institutional Animal Care and Use Committee (IACUC) and complied with the National Institutes of Health's guidelines for the care and use of laboratory animals (NIH Publication 85-23, revised 1996). The University of Pennsylvania, with the supervision of the School of Veterinary Medicine, maintains a full-service vivarium facility operated by the University Laboratory Animal Research (ULAR) organization. The vivarium is directly adjacent to the laboratory's operating room suite.

IMR was induced in fourteen adult (30-40 kg) Dorset sheep, randomized by sex, raised for laboratory work following a well-established protocol[9,12]. There is no data regarding sex differences in sheep after IMR model development, and due to the small sample size of the groups, data from male and female animals were pooled as in previous studies[9]. Sheep were anesthetized with sodium thiopental (10-15 mg/kg) intravenously, intubated, and ventilated with isoflurane (1.5–2%) and oxygen. MI was induced by ligation of the second and third obtuse marginal branches of the circumflex coronary artery through a thoracotomy. IMR-presenting and -non-presenting sheep were randomly assigned to two groups to be sacrificed at 4 weeks or 8 weeks post-MI. Ten un-operated sheep were used as control. All sheep were euthanized by an overdose of KCl and sodium thiopental while under general anesthesia. The animals were pronounced dead only after electrocardiogram silence is demonstrated for 3-5 minutes and cardiac arrest was visibly confirmed. This technique has been approved by the University of Pennsylvania IACUC and is consistent with the recommendations of the American Veterinary Medical Association (AVMA) Guidelines on Euthanasia. MV anterior and posterior leaflets were collected and portioned to be flash-frozen for RNA and protein analysis, formalin-fixed for histology and immunohistochemistry (IHC) analysis, or processed for MVIC isolation.

RNA Isolation and PCR

Isolation of total mRNA for RNA-sequencing and real-time PCR was performed using the RNeasy mini kit (Qiagen, Valencia, CA). 30 mg of flash-frozen frozen tissue were homogenized with a TissueRuptor (Qiagen). RNA concentration was measured on a Qubit 2.0 fluorometer (Invitrogen, Whaltham, MA). RNA integrity assessment was performed on an Agilent Bioanalyzer 2100 using a Nano 6000 assay kit (Agilent Technologies, Santa Clara, CA). All samples displayed RINs > 8.6. For PCR, 100ng of RNA were retro-transcripted with a Maxima H Minus cDNA Synthesis Master Mix kit (Thermo Scientific, Whaltham, MA). Gene expression was measured by Sybr green-based assays on a Piko Real real-time PCR apparatus (Thermo). The primers used are included in **Table II**. GAPDH and hypoxanthine-guanine phosphoribosyltransferase (HPRT) were used as housekeeping genes. Results were calculated with the $2^{-\Delta\Delta CT}$ method and expressed as a percentage of expression in control sheep.

RNA Sequencing (RNA-seq)

RNA library construction and transcriptome sequencing

RNA library preparation and sequencing were performed at Novogene (Sacramento, CA) as recently published[9]. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA), and index codes were added to attribute sequences to each sample. Library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina platform and 125 bp/150 bp paired-end reads were generated.

Data Preprocessing

Raw reads (in FASTQ format) were processed with Perl scripts. Low quality reads and those containing adapters or poly-N sequences were removed from raw data. The Ovis aries v3.1 reference genome was obtained from Ensembl (release 76). The index of the reference genome was built using Bowtie (v2.2.3) and paired-end reads were aligned to the reference genome using TopHat (v2.0.12). HTSeq (v0.6.1) was used to count the reads numbers mapped to each gene.

Differential Expression Analysis and Annotation

Differential expression analysis of two conditions/groups (>4 biological replicates per condition) was performed using the DESeq R package (1.18.0) using a model based on the negative binomial distribution. The resulting p-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted p-value < 0.1 found by DESeq were assigned as differentially expressed. Annotation was performed with BioConductor[13]. Genes were annotated with NCBI Entrez IDs, gene symbols, human homolog Entrez IDs, and human homolog gene symbols using biomaRt[14].

Overrepresentation Analysis of Differentially Expressed Genes

Overrepresentation analysis was performed using Enrichr[15,16] with pre-defined gene sets from NIH BioPlanet[17].

Protein-Protein Interaction (PPI) Analysis

PPI visualization of the differentially expressed sequences in post-MI MVs with IMR vs. post-MI MVs without IMR was performed using Genes2Networks[18] (G2N) with a minimum path length of two and the tool's default PPI network selection.

Enrichment Analysis of Differentially Expressed Genes

Gene set enrichment analysis (GSEA) was performed comparing the complete list of differentially expressed sequences, ranked by log2 fold change to the gene set using a Kolmogorov-Smirnov statistic and the pathways in BioPlanet.

Histology and IHC

Standard histology was performed in 5μm MV sections (n≥4/group and leaflet type) were stained with the following stains/antibodies: Movat's Pentachrome for visualization of collagen (yellow-orange), glycosaminoglycans (GAGs; light blue), and elastin (dark purple); α-smooth muscle actin (αSMA) as a marker of activated MVIC phenotype; the cell division protein Ki67 as a marker of proliferation; osteopontin (OPN), as a marker of bone morphogenic protein receptor (BMPR)-dependent signaling, and CD45 as a marker of cells from hematopoietic origin. The antibodies and concentration used, as well as negative controls for each antibody, are shown in the Supplemental Material within the Major Resource table **(Table I)** and **Figure II**.

Protein isolation and Western Blot

Isolation of protein for western blot was performed using RIPA buffer supplemented with a proteinase inhibitor (cOmplete, Roche, Palo Alto, CA) and a phosphatase inhibitor (Halt™, Thermo). 30 mg of flash-frozen frozen tissue were homogenized with a TissueRuptor (Qiagen). 30μg of protein were used to perform western blot using standard procedures alongside appropriate positive controls. The antibodies and conditions used are included in the Supplemental Material in the Major Resources Table **(Table I)**. The positive controls and conditions used are shown in **Figure IV**.

Cell Isolation and Culture

Interstitial cells were isolated from freshly explanted MV leaflets. The isolation protocol favors the culture of adherent cells and endothelial cells are removed in the process. Leaflet cusps were minced and digested with 1 mg/ml collagenase type 2 (Worthington Biochemical Corporation, Lakewood, NJ) and 100IU/mL hyaluronidase (Worthington) in complete growth medium for 16h-24h at 37°C. After digestion, the cell suspension was pelleted in a centrifuge (5min, 2220 rcf). Cells were cultivated in complete growth medium (Advanced DMEM containing 4.5g/ml glucose, supplemented with 10% FBS, 4 mM L-glutamine, 1% penicillin/streptomycin). Cryorecovered MVICs at passages 3-6 were used for all experiments. MVICs were treated for 24h with or without 10ng/ml TGFB1 or 50ng/ml bone morphogenic protein (BMP)-4. Conditions were tested in duplicate and three sets of experiments were performed. At the end of the treatment, total RNA was isolated with a Qiagen RNeasy kit. Gene expression was measured as described above for MV tissue with GAPDH as a housekeeping gene. Results were calculated with the $2^{-\Delta\Delta CT}$ method and expressed as a percentage of expression in untreated cells from control sheep. Separate plates were used for protein analysis and isolated as described above for MV tissue.

Collagen contraction assay

Collagen contraction assay was performed followed manufacturer instructions (Cellbiolabs). MVICs were seeded in collagen matrix mixture at a 4 million cell/ml concentration. Contraction of the collagen disc was assessed after 24h of treatment.

Data analysis

PCR results are reported as means ± standard error. No data were excluded from the analysis. Due to the small sample size, the normality and variance were not tested to determine whether the applied parametric tests were appropriate. Statistical analysis was performed by two-way or one-way ANOVA followed by Tukey's post hoc test, or Student's t-test (IMR vs. no IMR), as appropriate. For all analyses, p-values were two-sided and a $p<0.05$ was considered significant. All data were analyzed with Prism software (GraphPad Software, San Diego, CA).

Table II. Primer Sequences (*Ovis aries*)

Gene	Forward primer 3'-5'	Reverse primer 3'-5'
GAPDH	ACAGTCAAGGCAGAGAACGG	CCAGCATCACCCCACTTGAT
HPRT	ACACAGCTGTATTTCTTTTTCAGA	ACATCTCGAGCCAGTCGTTC
COL1A1	GGCAGGAAGCTCAAGTCGTA	TGTAAGTCCCCCAACCCTGT
ELN	CCAGGAGCAATACCAGGCG	GGCCCCTTCCATGAGATAAAC
SMAD6	CATTGGCTGCCTCCCATCAT	TGTTTGCGCAACAATCAGCG
SMAD7	AGACTGTCCAGATGCTGTGC	CAGTAGAGCCTCCCCACTCT
ID2	CCTTCAGTCCAGTGAGGTCCG	GATGGTGGGGTGCGAGTCTA
OPN	CTGATTTTCCCACTGACATT	GGTGATGTCCTCGTCTGTA
OC	AGCTCATCACAGTCAGGGTTG	AGCGAGGTGGTGAAGAGA
RUNX2	GCCTGGGGTCTGTAATCTGA	CGCATTCCTCATCCCAGTAT
SOX9	CTCTGGAGACTGCTGAACGAG	GCCGTTCTTCACCGACTTCC
BMP4	CCTTGTTTTCTGTCAAGACACCAT	GCAGGACTTGGCATAATAAAACGA
BMPRII	GTTGGAATTAGTTCGCCCGC	TGGCCAGACAGCTAACACAG
TGFBRII	CGGTCTCTTAGCACGCTGT	CGTGGGAGAAGTGAAGGACTAC
CD45	TGGCATTTGGCTTTGCCTTC	CTTAACATCGAGCGTTGCGG

Supplemental Results

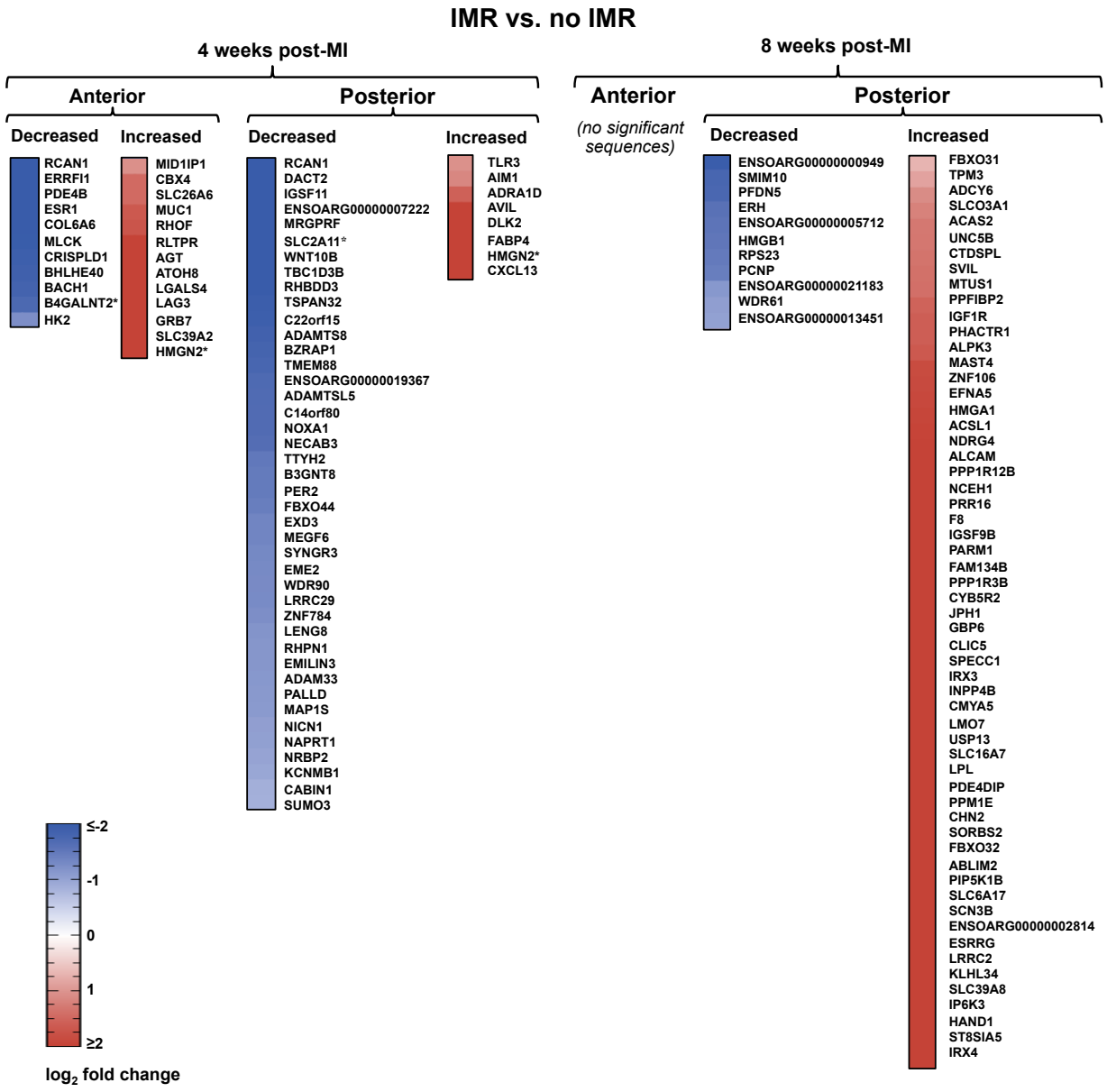


Figure I: RNA sequencing results. Log 2 fold changes for genes with a p-value of ≤ 0.1 (Wald test with Benjamini-Hochberg correction) in the comparisons “IMR vs. no IMR” at each time point for both anterior and posterior cohorts. Asterisks indicate gene names that were not present in the original annotation and instead found through BLAST searches on the *Ovis aries* genome.

Table III. Gene names for symbols in Figure S1. Genes with a p-value of ≤ 0.1 in the comparisons "IMR vs. no IMR" at each time point for both anterior and posterior cohorts.

Symbol	Gene Name	Change vs. no IMR
ABLIM2	actin binding LIM protein family member 2	8 week, posterior, increased
ACAS2	acyl-CoA synthetase short chain family member 1	8 week, posterior, increased
ACSL1	acyl-CoA synthetase long chain family member 1	8 week, posterior, increased
ADAM33	ADAM metallopeptidase domain 33	4 week, posterior, decreased
ADAMTS8	ADAM metallopeptidase with thrombospondin type 1 motif 8	4 week, posterior, decreased
ADAMTSL5	ADAMTS like 5	4 week, posterior, decreased
ADCY6	adenylate cyclase 6	8 week, posterior, increased
ADRA1D	alpha-1D adrenergic receptor	4 week, posterior, increased
AGT	angiotensinogen	4 week, anterior, increased
AIM1	crystallin beta-gamma domain containing 1	4 week, posterior, increased
ALCAM	activated leukocyte cell adhesion molecule	8 week, posterior, increased
ALPK3	alpha kinase 3	8 week, posterior, increased
ATOH8	atonal bHLH transcription factor 8	4 week, anterior, increased
AVIL	advillin	4 week, posterior, increased
B3GNT8	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 8	4 week, posterior, decreased
B4GALNT2	beta-1,4-N-acetyl-galactosaminyltransferase 2	4 week, anterior, decreased
BACH1	BTB domain and CNC homolog 1	4 week, anterior, decreased
BHLHE40	basic helix-loop-helix family member e40	4 week, anterior, decreased
BZRAP1	TSPO associated protein 1	4 week, posterior, decreased
C14orf80	tubulin epsilon and delta complex 1provided	4 week, posterior, decreased
C22orf15	chromosome 17 C22orf15 homolog	4 week, posterior, decreased
CABIN1	calcineurin binding protein 1	4 week, posterior, decreased
CBX4	chromobox 4	4 week, anterior, increased
CHN2	chimerin 2	8 week, posterior, increased
CLIC5	chloride intracellular channel 5	8 week, posterior, increased
CMYA5	cardiomyopathy associated 5	8 week, posterior, increased
COL6A6	collagen type VI alpha 6 chain	4 week, anterior, decreased
CRISPLD1	cysteine rich secretory protein LCCL domain containing 1	4 week, anterior, decreased
CTDSPL	CTD small phosphatase like	8 week, posterior, increased
CXCL13	C-X-C motif chemokine ligand 13	4 week, posterior, increased
CYB5R2	cytochrome b5 reductase 2	8 week, posterior, increased
DACT2	dishevelled binding antagonist of beta catenin 2	4 week, posterior, decreased
DLK2	delta like non-canonical Notch ligand	4 week, posterior, increased
EFNA5	ephrin A5	8 week, posterior, increased
EME2	essential meiotic structure-specific endonuclease subunit 2	4 week, posterior, decreased
EMILIN3	elastin microfibril interfacer 3	4 week, posterior, decreased
ENSOARG00000000949	60S ribosomal protein L21-like	8 week, posterior, decreased
ENSOARG00000002814	novel gene	8 week, posterior, increased
ENSOARG00000005712	novel gene	8 week, posterior, decreased
ENSOARG00000007222	novel gene	4 week, posterior, decreased
ENSOARG00000013451	heterogeneous nuclear ribonucleoprotein A3-like	8 week, posterior, decreased
ENSOARG00000019367	novel gene	4 week, posterior, decreased
ERH	ERH mRNA splicing and mitosis factor	8 week, posterior, decreased
ERRFI1	ERBB receptor feedback inhibitor 1	4 week, anterior, decreased
ESR1	estrogen receptor 1	4 week, anterior, decreased
ESRRG	estrogen related receptor gamma	8 week, posterior, increased

(Table III continues)

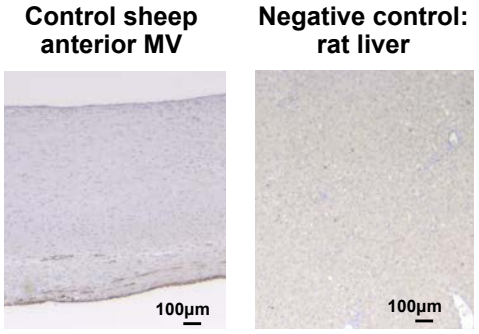
Symbol	Gene Name	Change vs. no IMR
EXD3	exonuclease 3'-5' domain containing 3	4 week, posterior, decreased
FABP4	fatty acid binding protein 4, adipocyte	4 week, posterior, increased
FAM134B	reticulophagy regulator 1	8 week, posterior, increased
FBXO31	F-box only protein 31	8 week, posterior, increased
FBXO32	F-box only protein 44	8 week, posterior, increased
FBXO44	F-box only protein 44	4 week, posterior, decreased
F8	coagulation factor VIII	8 week, posterior, increased
GBP6	guanylate binding protein family member 6	8 week, posterior, increased
GRB7	growth factor receptor bound protein 7	4 week, anterior, increased
HMGA1	high mobility group AT-hook 1	8 week, posterior, increased
HMGB1	high mobility group box 1	8 week, posterior, decreased
HMGN2	high mobility group nucleosomal binding domain 2	4 week, anterior, increased, 4 week, posterior, increased
HAND1	heart and neural crest derivatives expressed 1	8 week, posterior, increased
HK2	hexokinase 2	4 week, anterior, decreased
IGF1R	insulin like growth factor 1 receptor	8 week, posterior, increased
IGSF11	immunoglobulin superfamily member 11	4 week, posterior, decreased
IGSF9B	immunoglobulin superfamily member 9B	8 week, posterior, increased
INPP4B	inositol polyphosphate-4-phosphatase type II B	8 week, posterior, increased
IP6K3	inositol hexakisphosphate kinase 3	8 week, posterior, increased
IRX3	iroquois homeobox 3	8 week, posterior, increased
IRX4	iroquois homeobox 4	8 week, posterior, increased
JPH1	junctophilin 1	8 week, posterior, increased
KCNMB1	potassium calcium-activated channel subfamily M regulatory beta subunit 1	4 week, posterior, decreased
KLHL34	kelch like family member 34	8 week, posterior, increased
LAG3	lymphocyte activating 3	4 week, anterior, increased
LENG8	leukocyte receptor cluster member 8	4 week, posterior, decreased
LGALS4	galectin 4	4 week, anterior, increased
LMO7	LIM domain 7	8 week, posterior, increased
LPL	lipoprotein lipase	8 week, posterior, increased
LRRC2	leucine rich repeat containing 2	8 week, posterior, increased
LRRC29	F-box/LRR-repeat protein 2	4 week, posterior, decreased
MAP1S	microtubule associated protein 1S	4 week, posterior, decreased
MAST4	microtubule-associated serine/threonine-protein kinase 4	8 week, posterior, increased
MEGF6	multiple EGF like domains 6	4 week, posterior, decreased
MID1IP1	MID1 interacting protein 1	4 week, anterior, increased
MLCK	myosin light chain kinase	4 week, anterior, decreased
MRGPRF	MAS related GPR family member F	4 week, posterior, decreased
MTUS1	microtubule associated scaffold protein 1	8 week, posterior, increased
MUC1	mucin 1	4 week, anterior, increased
NAPRT1	nicotinate phosphoribosyltransferase	4 week, posterior, decreased
NCEH1	neutral cholesterol ester hydrolase 1	8 week, posterior, increased
NDRG4	NDRG family member 4	8 week, posterior, increased
NECAB3	N-terminal EF-hand calcium binding protein 3	4 week, posterior, decreased
NICN1	nicolin 1	4 week, posterior, decreased
NOXA1	NADPH oxidase activator 1	4 week, posterior, decreased
NRBP2	nuclear receptor binding protein 2	4 week, posterior, decreased
PALLD	palladin	4 week, posterior, decreased
PARM1	prostate androgen-regulated mucin-like protein 1	8 week, posterior, increased

(Table III continues)

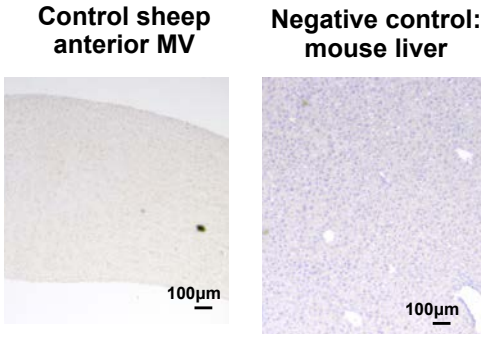
Symbol	Gene Name	Change vs. no IMR
PCNP	PEST proteolytic signal containing nuclear protein	8 week, posterior, decreased
PDE4B	phosphodiesterase 4B	4 week, anterior, decreased
PDE4DIP	phosphodiesterase 4D interacting protein	8 week, posterior, increased
PER2	period circadian regulator 2	4 week, posterior, decreased
PFDN5	prefoldin subunit 5	8 week, posterior, decreased
PHACTR1	phosphatase and actin regulator 1	8 week, posterior, increased
PIP5K1B	phosphatidylinositol-4-phosphate 5-kinase type 1 beta	8 week, posterior, increased
PPFIBP2	PPFIA binding protein 2	8 week, posterior, increased
PPM1E	protein phosphatase, Mg2+/Mn2+ dependent 1E	8 week, posterior, increased
PPP1R12B	protein phosphatase 1 regulatory subunit 12B	8 week, posterior, increased
PPP1R3B	protein phosphatase 1 regulatory subunit 3B	8 week, posterior, increased
PRR16	proline rich 16	8 week, posterior, increased
RCAN1	regulator of calcineurin 1	4 week, anterior, decreased 4 week, posterior, decreased
RHBDD3	rhomboid domain containing 3	4 week, posterior, decreased
RHOF	ras homolog family member F, filopodia associated	4 week, anterior, increased
RHPN1	rhophilin Rho GTPase binding protein 1	4 week, posterior, decreased
RLTPR	RGD motif, leucine rich repeats, tropomodulin domain and proline-rich containing	4 week, anterior, increased
RPS23	ribosomal protein S23	8 week, posterior, decreased
SCN3B	sodium voltage-gated channel beta subunit 3	8 week, posterior, increased
SLC16A7	solute carrier family 16 member 7	8 week, posterior, increased
SLC2A11	solute carrier family 2 member 11	4 week, posterior, decreased
SLC26A6	solute carrier family 26 member 6	4 week, anterior, increased
SLC39A2	solute carrier family 39 member 2	4 week, anterior, increased
SLC39A8	solute carrier family 39 member 8	8 week, posterior, increased
SLC6A17	solute carrier family 6 member 17	8 week, posterior, increased
SLCO3A1	solute carrier organic anion transporter family member 3A1	8 week, posterior, increased
SMIM10	small integral membrane protein 10	8 week, posterior, decreased
SORBS2	sorbin and SH3 domain containing 2	8 week, posterior, increased
SPECC1	sperm antigen with calponin homology and coiled-coil domains 1	8 week, posterior, increased
ST8SIA5	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 5	8 week, posterior, increased
SUMO3	small ubiquitin like modifier 3	4 week, posterior, decreased
SVIL	supervillin	8 week, posterior, increased
SYNGR3	synaptogyrin 3	4 week, posterior, decreased
TBC1D3B	TBC1 Domain Family Member 3B	4 week, posterior, decreased
TLR3	toll like receptor 3	4 week, posterior, increased
TMEM88	transmembrane protein 88	4 week, posterior, decreased
TPM3	tropomyosin 3	8 week, posterior, increased
TSPAN32	tetraspanin 32	4 week, posterior, decreased
TTYH2	tweety family member 2	4 week, posterior, decreased
UNC5B	unc-5 netrin receptor B	8 week, posterior, increased
USP13	ubiquitin specific peptidase 13	8 week, posterior, increased
WDR61	WD repeat domain 61	8 week, posterior, decreased
WDR90	WD repeat domain 90	4 week, posterior, decreased
WNT10B	Wnt family member 10B	4 week, posterior, decreased
ZNF106	zinc finger protein 106	8 week, posterior, increased
ZNF784	zinc finger protein 784	4 week, posterior, decreased

Immunostaining negative controls

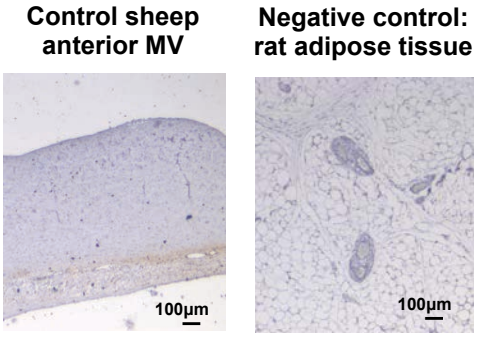
αSMA: Abcam #ab7817, 0.3µg/ml



CD45: Abcam #ab10559, 1µg/ml



OPN: Abcam #ab8448, 1µg/ml



Ki67: Abcam #ab15580, 1µg/ml

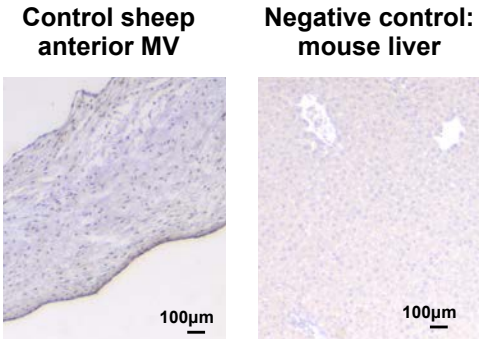


Figure II: Negative controls for antibodies used for immunostaining.

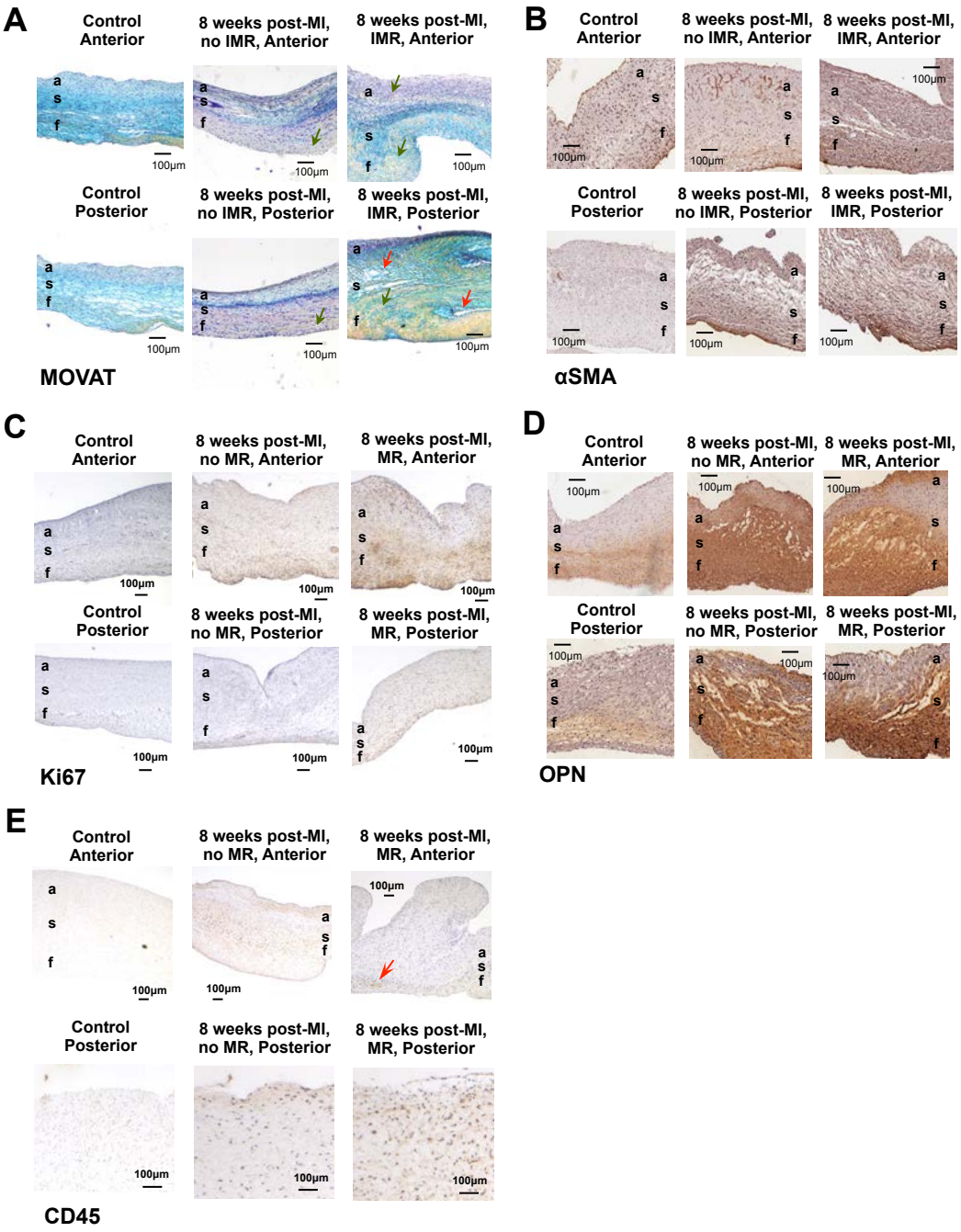


Figure III: Additional immunostaining images **(A)** Representative MOVAT staining of control and post-MI sheep at 8 week post MI, with and without IMR, anterior and posterior MV leaflets. (a) zona atrialis, (s) zona spongiosa and (f) zona fibrosa. Green arrow point at zones of diffuse extracellular matrix. Red arrow point at zones with increased interfibrillar spaces. **(B)** Representative IHC of α-smooth muscle actin (SMA), anterior and posterior MV leaflets. **(C)** Representative IHC of the cell cycle marker Ki67, anterior and posterior MV leaflets. **(D)** Representative IHC of the osteogenic marker osteopontin (OPN), anterior and posterior MV leaflets. **(E)** Representative IHC of CD45 in sheep anterior and posterior MV leaflets. Red arrow indicates clustering of CD45 positive cells in the fibrosa interstitium.

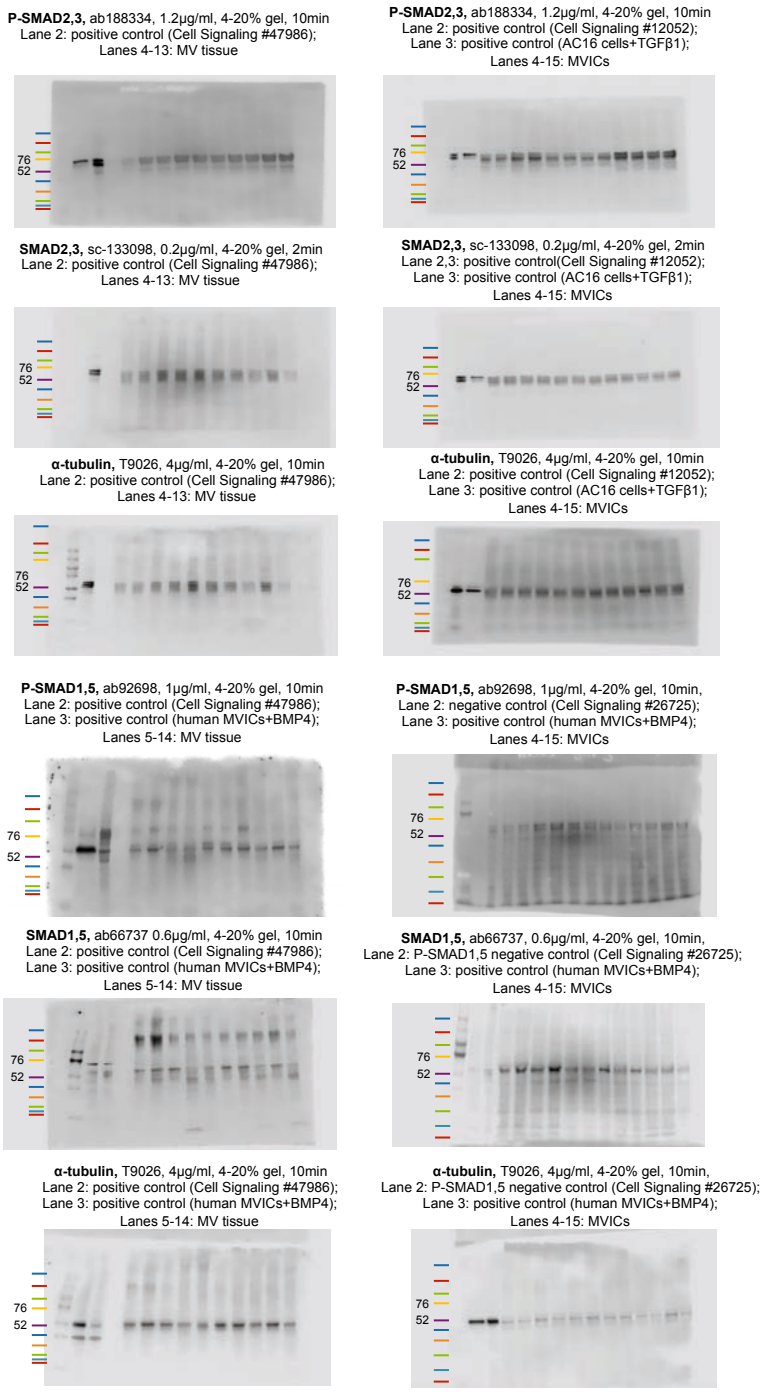


Figure IV: Uncropped representative blots corresponding to each antibody used for Western Blot analysis, indicating % agarose of gels, time of exposure for image capture, and positive controls used. Amersham™ ECL™ Rainbow™ Marker (Sigma #GERPN800E) was used as molecular weight marker.